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Suspended bed chromatography, a new approach in downstream processing

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Abstract

A new technique in downstream processing, suspended bed chromatography has been developed. This hybrid technique exploiting the benefits of batch adsorption and the process advantages of an enclosed column system can be carried out using established contactors and adsorbents. A 44 cm I.D. IsoPak column and the anion-exchange cellulose Express-Ion Exchanger Q were used in the purification of ovalbumin from hen-egg white. After suspension of 16.25 kg Express-Ion Q in 500 l of feedstock containing 5 g protein/l, adsorption was effected by recirculation of the suspension using the IsoPak slurry preparation station. Protein-loaded adsorbent was collected in the IsoPak column unit, where it was washed and protein desorbed using gradient elution at a flow-rate of 300 cm/h. The entire process was complete in under 3 h. With the introduction of pump-packed column systems and the availability of mechanically strong adsorbents suitable for column separations, suspended bed chromatography offers a new approach to downstream processing and provides a less challenging alternative to batch separations.

Keywords: Suspended bed chromatography; Adsorption; Preparative chromatography; Proteins; Ovalbumin

1. Introduction

The downstream processing of commercially important biopolymers including, proteins, peptides, nucleic acids and carbohydrates from various sources is gaining importance. Low-pressure ion-exchange chromatography is a routine technique in these applications. As the processes are intensified so the demands on the separation increase in terms of separation efficiency, throughput and process economics. In order to address these demands we have seen the gradual introduction of new adsorbents and new contactors over the past few years.

Protein separations can be carried out in either a positive or negative ion-exchange step, where either the target or contaminants are retained, respectively [1]. In each case the separation could be carried out using a batch- or column-based contacting system and we have compared and reported each of these techniques at process-scale elsewhere [2,3]. On the premise that large-scale column processes typically operate at flow-rates of <200 cm/h then processing time becomes a major driver in the throughput calculations. For an ion-exchange column packed to a bed height of 20 cm, a flow-rate of 200 cm/h equates to ca. 10 bed volumes/h. The processing issue then becomes how many bed volumes of

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feedstock should you load before the adsorption process becomes unattractive or uneconomic? For example, in a typical 8 h shift you could load ca. 80 bed volumes, i.e., 2 m^3 feedstock through a 25 l bed. In applications of this sort, where a large volume of feedstock requires contacting with a relatively small mass of ion exchanger, or in systems where either the adsorption kinetics or pressure–flow performance of the adsorbent–adsorbate system preclude the use of high flow-rates, then the adsorption stage of the chromatography may take many hours. This has obvious implications on the time for completing this unit operation. In systems where the feedstream contains unstable components or could be subject to enzymatic degradation, for example, feedstock storage should be minimised as much as possible, in order to maximise the recovery of target. For any of these reasons batch chromatography may be an appropriate alternative to packed bed column chromatography since the adsorption is typically complete in 30–60 min, regardless of scale [2,3]. It should be noted that traditional batch chromatography while a simple equilibrium process, requires specialised filtration or centrifugation equipment to facilitate efficient and effective recovery of adsorbent and subsequent elution of target [3]. Furthermore, batch processes are labour intensive, may be difficult to automate and problematic to validate [3], key drivers for the use of contained packed bed column systems [4].

In order to reconcile the advantages of batch with the benefits of column, we reported a study where the products of a batch adsorption were packed into a chromatography column [3]. While this approach goes partway to resolving the issues discussed above it still required the harvesting of the adsorbent from the depleted feedstock following the batch step, prior to column packing, and accordingly required specialised centrifugation equipment.

Recent innovations in column design have resulted in the availability of adjustable volume columns designed for rapid pump-packing of a slurry of adsorbent to facilitate rapid bed consolidation and also are configured to permit rapid pump unpacking using in situ slurry preparation within the column unit itself [5,6]. We have recently reported the use of such a column design for the process-scale anion-exchange separation of hen egg-white proteins in a 25 l IsoPak column using Express-Ion Exchanger Q at flow-rates of up to 300 cm/h [7]. In this study we reported that the 25-l bed could be packed within 10 min and unpacked in a similar time, without any need for column disassembly which has usually been necessary in order to manually excavate spent adsorbent [7]. The IsoPak unit which we evaluated comprised a 50 cm×44 cm I.D. column assembly and an associated recirculating slurry preparation station. In our investigation we adjusted the column height to 16 cm and packed a 30% (w/v) slurry of Express-Ion Q into the column. The slurry was maintained throughout preparation and packing by recirculation through the slurry preparation unit at a flow-rate of 30–40 l/min.

In the present study, we extended our previous work on IsoPak and evaluated the application of this unit for both batch adsorption and column desorption in a single-closed system. We report a new technique, suspended bed chromatography, which is a hybrid process exploiting the benefits of batch adsorption with the process advantages of an enclosed column system. Furthermore it is a technique which requires neither specialised equipment nor specialised adsorbents.

2. Experimental

2.1. Materials

Cell debris remover (CDR) and Express-Ion Q were obtained from Whatman (Maidstone, UK). An IsoPak column (50 cm×44 cm I.D.) and associated recirculating slurry preparation station equipped with a 750-l vessel was obtained from Millipore (Stonehouse, UK). Tris(hydroxymethyl)aminomethane (Tris) was obtained from Merck (Poole, UK). All other chemicals were of analytical reagent grade. Fresh large hen eggs were obtained from Barradale Farms (Headcorn, UK).

2.2. Feedstock preparation

Egg-whites (55 l) were separated from 1440 fresh hen eggs and diluted to 10% (v/v) with 0.025 *M* Tris–HCl buffer (pH 7.5). The egg-white suspension was clarified using a total of 35 kg of pre-equili-

brated CDR in a batch mode. Spent CDR was removed by centrifugation through a 1.6×0.6 mm slotted screen (EHR 500 basket centrifuge, Robatel and Mulatier, Lyons, France) and the sample clarified through a Grade 541 filter paper (Whatman) [3]. The clear solution (550 l) containing 5 mg/ml of total protein was used for chromatography on Express-Ion Q.

2.3. Suspended bed chromatography

Express-Ion Q (16.25 kg) was equilibrated with 0.025 *M* Tris–HCl buffer (pH 7.5) and collected by centrifugation through a 1.6×0.6 mm slotted screen (EHR 500 basket centrifuge). Egg-white feedstock (500 l) was transferred to the IsoPak slurry preparation vessel (750 l), and the equilibrated Express-Ion Q added to the feedstock. The ion exchanger was maintained in suspension by continuous recirculation of the slurry through the IsoPak slurry preparation unit at a flow-rate of 30–40 l/min. Adsorption was continued in suspended bed mode for 30 min.

The height of the 44 cm I.D. IsoPak column was preset to 16.3 cm (ca. 24.8 l) and the adsorbent–adsorbate suspension pumped through the column in upflow from the slurry tank at a pressure of ca. 10 p.s.i. (1 p.s.i.=6894.76 Pa) according to the column manufacturer's guidelines. During this stage of column packing, depleted feed was exhausted to waste. Bed consolidation was continued until ca. 100 l suspension remained at which time, the exhaust liquid was recirculated through the system to ensure all Express-Ion was packed into the column, and a further 30 l of depleted feed exhausted to waste. Additional pre-equilibrated Express-Ion Q (2.8 kg) was suspended in the residual 70 l depleted feed and this slurry was pumped into the column to complete column packing. The packed column of Express-Ion Q (16.3 cm×44 cm I.D.) had a volume of ca. 24.8 l and a packing density of 0.229 kg/l. Non-bound material was removed by washing with 0.025 *M* Tris–HCl buffer (pH 7.5) (150 l). Bound material was eluted using a linear gradient of 0–0.5 *M* NaCl in 0.025 *M* Tris–HCl buffer (pH 7.5) (400 l). All procedures were carried out in upflow at room temperature (15–20°C). Flow-rate was maintained at 300 cm/h during the column operations.

Following suspended bed chromatography the IsoPak column was pump-unpacked, without disassembly.

2.4. Assays

Pooled fractions at various stages of chromatography were assayed for total protein and ovalbumin content by fast protein liquid chromatography (FPLC) [8].

3. Results and discussion

The non-regenerated microgranular cellulose matrix upon which the Whatman ion-exchange product ranges are based is chemically and physically robust lending itself to mechanical pumping and agitation under suitable conditions [9]. This feature has enabled their application in batch processes where significant numbers of slurry transfers are required [2,3]. This facilitates their use in pump-packed columns and we have recently reported their use in an IsoPak column system, where Express-Ion Q supported flow-rates of >300 cm/h following pump-packing of a 25-l bed [7]. In the present study we set out to investigate the opportunity of combining the benefits of batch, in terms of a short overall time for adsorption independent of flow-rate, with the advantages of column elution in terms of resolution, recovery and containment. As a direct consequence of the IsoPak column design [6], we were able to use the recirculating slurry preparation station as a batch contactor, in which the adsorbent bed was suspended and well mixed by recirculation of the slurry, and which was furthermore contained in a closed loop. Following adsorption, the protein-loaded ion exchanger could be removed from the slurry directly in the column unit using the upper bed support as a filter to retain the media with concomitant bed consolidation under constant packing pressure. This is a single-stage unit operation and replaces the traditional filtration or centrifugation processes that are routinely used in batch chromatography [2,3], often requiring the use of an ancillary second-stage contactor. It offers the added benefit of containment of the adsorbent in a closed system, a significant disadvantage of traditional batch processes that may be typically regarded as open systems [3]. Once the

Table 1
Protein mass balance data during the suspended bed chromatography of hen egg-white proteins using Express-Ion Q

Stage of chromatography	Feedstock Total protein (kg)	Total protein (kg) In mobile phase	Adsorbed to Express-Ion Q
Loading	2.520	–	–
Wash	–	1.403	1.117
Elution	–	1.148	–

bed was consolidated using the IsoPak pump-packing protocol [6], then the Express-Ion Q was washed and bound protein gradient eluted using conventional column procedures [7].

The protein mass balance data for the suspended bed chromatography of hen egg-white protein using Express-Ion Q are summarised in Table 1 and the chromatogram represented in Fig. 1. It has previously been reported that Express-Ion Q has an ovalbumin binding capacity of ca. 60–70 mg/ml [7,10]. Hen egg-white contains 63.8% (w/w) ovalbumin [11] and in the present study we loaded the Express-Ion Q with ca. 0.1 kg protein/l packed bed, i.e., levels of ovalbumin similar to the anticipated maximum dynamic capacity of the packed bed, and directly equivalent to our earlier column-based studies using the same IsoPak system [7]. Under these conditions ca. 1.1 kg of protein bound to the Express-Ion Q during the suspended bed adsorption with ca. 100% (w/w) recovery during gradient elution in the column (Table 1). FPLC analysis of the desorbed protein demonstrated it to be predominantly ovalbumin, with some ovomucoid and ovoglobulin content, observations in keeping with those previously reported for Express-Ion Q [7,10].

The process times for the suspended bed chromatography of hen egg-white proteins using Express-Ion Q is summarised in Table 2. The data demonstrate completion of the entire chromatographic process within 3 h. It should be noted that these times do not include feedstock preparation which is clearly an application specific upstream process which is a constant parameter regardless of the mode of contacting, i.e., batch or packed bed. However in this particular application feedstock preparation was completed within the working day [3]. We believe that the only variable associated with scale is the column packing time and this would correlate with

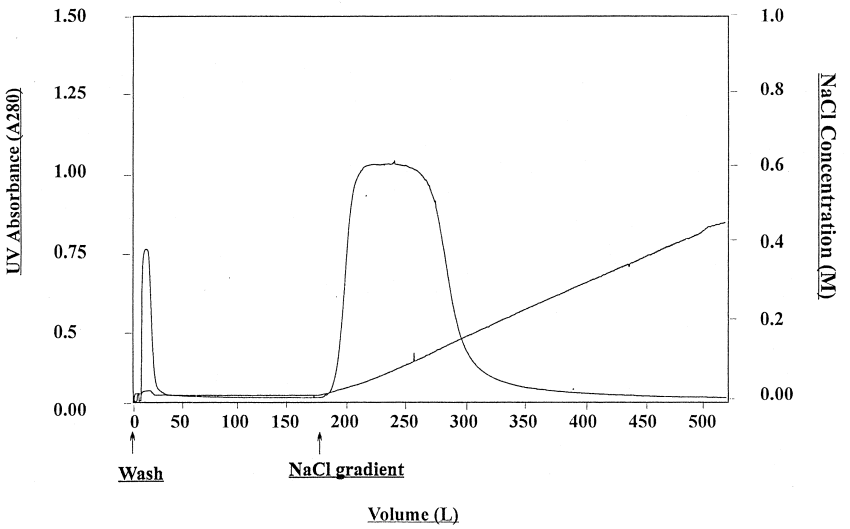

Fig. 1. Process-scale chromatography of hen egg-white proteins on Express-Ion Q in an IsoPak column (16.3 cm×44 cm ID) using 0.025 *M* Tris–HCl buffer (pH 7.5) at a flow-rate of 300 cm/h following suspended bed adsorption.

Table 2
Process times for the suspended bed chromatography of hen egg-white proteins using Express-Ion Q

Stage of process	Stage time (min)
Adsorption	30
Column packing	60
Wash	20
Elution	50
Unpacking	10
Total process time	170

the slurry consistency following suspended bed adsorption. We have reported that bed consolidation is carried out at constant pressure in the IsoPak column [7] and this approach was adopted following suspended bed adsorption. In this case, however, the slurry consistency is low, hence bed formation takes longer due to the large volume of depleted feed which must pass through the upper bed support. This may be partially compensated for since proportionally faster flow-rates can be supported during the early stages of bed consolidation since the build-up of back-pressure due to the media is slower than during conventional column packing which uses more concentrated slurries. Consequently column packing of an even more dilute slurry would take longer than the 60 min we report here, but is unlikely to be directly proportioned to volume for the reasons mentioned above. Provided the column diameter was chosen to maintain similar bed height to that reported here, then all other stages of the process should remain constant regardless of scale.

Process-time in all stages of suspended bed chromatography will nevertheless be influenced by media selection. For example, the kinetics of adsorption will affect capture efficiency during the adsorption stage, and so dictate the time for this step. The pressure/flow characteristics of the medium will dictate the process time for all in-column operations, and the desorption kinetics may influence the elution stage in terms of product recovery, purity and concentration. We have reported the efficient packed column use of Express-Ion Q in a similar application at a flow-rate of 300 cm/h [7]. Assuming the adsorbent has a voidage of 75% (v/v) then under these conditions in a packed bed of $<$20 cm the maximum contact time between adsorbent and adsorbate will be ca. 3 min. If diffusion kinetics for a particular adsorbent are limiting then capture efficiency of the adsorbent for the adsorbate during a single pass through a column may be inefficient precluding its economic use at such a high flow-rate. In such circumstances the necessary reduction in flow-rate would increase the process time which may have a negative impact on the process economics. On the other hand, batch being a simple equilibrium process, offers a longer contact time between adsorbent and adsorbate, i.e., 30–60 min, which may give rise to a better capture efficiency. It should be remembered that a simple equilibrium binding gives rise to slightly less favourable adsorption characteristics than observed in a packed bed. This may result in slightly lowered protein binding capacities for batch compared to a packed bed adsorption [3], although the use of a proportionally greater mass of adsorbent may offset this effect.

In our earlier work on the IsoPak column [7] we operated the unit in keeping with the manufacturer's suggestions, i.e., pack in upflow and operate in downflow. In such a typical application, bed height adjustment is made prior to packing, and then an excess of slurried adsorbent is pumped into the column unit, to ensure complete packing. Any residual adsorbent can be collected and made available for re-packing on a subsequent occasion. Feedstock is then applied to the packed bed until it is all used, or a specific breakthrough level is achieved, whereupon the bed is washed and product eluted.

In the case of batch adsorption, the adsorbent would typically be added to the feedstock and the mass used may vary according to the heterogeneity of each lot of feedstock. Assuming one can rapidly determine levels of specific components of the feedstock, it may be possible to continue adding adsorbent until a certain target level of components is achieved. This provides a high degree of flexibility in batch processing and may offer economic benefit. It does however create two potential obstacles for suspended bed applications. Firstly, while it is straightforward to determine column packing densities for ''clean'' adsorbents, it may be less predictable for a protein-loaded adsorbent, as the adsorbed proteins themselves have a volume, and may exert ill-defined forces on the adsorbent particles, which

could influence their packing properties. Secondly, how do you set a bed height, prior to packing an ill-defined volume of adsorbent, in order to ensure complete inclusion of all loaded adsorbent from the depleted feedstock. In the case of a positive chromatographic step, failure to pack all adsorbent in the column will directly result in recovery reduction and in the case of a negative step, the depleted feed will need further clarification to remove residual suspended adsorbent. Notwithstanding each of these obstacles, for suspended bed chromatography to operate effectively they must each be overcome, to ensure complete packing of all adsorbent in the column prior to the column-based stages of the separation.

In order to ensure packing of all the adsorbent in the column, the protocol described above was developed. Firstly, the operator needs to estimate the packed column volume in order to preset a bed height, and we suggest oversizing the volume by ca. 5–15% (v/v). This will likely create a void space at the base of the column following the packing operation. The suspended adsorbent was passed through the bed, exhausting 80% (v/v) leading to a volume of 100 l which was recirculated at constant pressure to ensure all suspended adsorbent was introduced into the column unit. At this stage a void may be present at the base of the pressurised column, although it may be obscured from view by the bottom bed support assembly. Whilst maintaining pressure, by recirculating 70 l of depleted feed through the system we resuspended fresh Express-Ion Q in the recirculating depleted feed and continued packing until the bed was fully consolidated, according to standard operating procedures for this unit.

Following the void-filling operation, all subsequent column operations, i.e., wash and elution were carried out in upflow to ensure that none of the unbound or desorbed material contacted the fresh adsorbent, which may have given rise to unwanted secondary interactions.

It should be noted that protein capacities determined as mass of protein per unit column volume, may no longer reflect the dynamic binding capacity of the ion exchanger. In traditional packed bed operations, the total mass of protein binding throughout the column is known and a capacity may be calculated, i.e., ca. 60–70 mg/ml for a similar system [7]. However in suspended bed chromatography, the volume of adsorbent used in adsorption is less than the volume packed into the column, due to the void-filling operation. Hence the effective protein capacity, in our case ca. 50 mg/ml is less than that which we have previously observed, but in this case we do not know the absolute packed volume of loaded adsorbent. In such situations it is recommended that the mass of protein binding be used as the prime measure of overall capacity rather than as mass per unit column volume.

Being a process based on batch adsorption, following the stage of product elution, the adsorbent may require cleaning either prior to re-use or for safe handling of used media prior to disposal in single-use applications. In our earlier packed bed study we detailed a CIP process using 0.5 *M* NaOH [7]. In suspended bed applications the used adsorbent following the elution stage requires removal from the column, either pre- or post-CIP, and the design of the IsoPak column enables rapid pump-unpacking, an operation taking ca. 10 min to complete. This operation has been discussed in more detail elsewhere [7]. This offers flexibility to the user for carrying out a validated CIP within the closed column assembly or for a batch CIP outside of the column unit.

In the present study we have reported a new technique in downstream processing, suspended bed chromatography, which is a hybrid technique exploiting the benefits of batch adsorption in terms of reduced contacting times for adsorption from large volumes of feedstock with all the process advantages of an enclosed column system. A key feature of this technique is that it does not require specialised equipment or specialised adsorbents, unlike other techniques such as fluidised or expanded beds. The only prerequisites are that the adsorbents are physically stable to the handling conditions required in batch chromatography, yet have hydraulic properties suitable for efficient column operation, and that the column unit is designed for in-situ pump-packing and pump-unpacking. To this end, the Millipore IsoPak column systems and the Whatman microgranular celluloses appear well suited to this type of chromatography as demonstrated in this example of the technique. Suspended bed chromatography lends

itself to single media-use applications since the column must be unpacked at the end of each process cycle, eliminating the need for in situ CIP and associated process validation, and is a less challenging alternative to traditional batch chromatography. Suspended bed chromatography offers an additional technique to the process chromatographer and should be considered as one option when designing a downstream process.

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